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Loss of Hsp70 in Drosophila Is Pleiotropic, With Effects on Thermotolerance, Recovery From Heat Shock and Neurodegeneration

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ABSTRACT

The heat-shock response is a programmed change in gene expression carried out by cells in response to environmental stress, such as heat. This response is universal and is characterized by the synthesis of a small group of conserved protein chaperones. In *Drosophila melanogaster* the Hsp70 chaperone dominates the profile of protein synthesis during the heat-shock response. We recently generated precise deletion alleles of the *Hsp70* genes of *D. melanogaster* and have used those alleles to characterize the phenotypes of *Hsp70*-deficient flies. Flies with *Hsp70* deletions have reduced thermotolerance. We find that *Hsp70* is essential to survive a severe heat shock, but is not required to survive a milder heat shock, indicating that a significant degree of thermotolerance remains in the absence of Hsp70. However, flies without *Hsp70* have a lengthened heat-shock response and an extended developmental delay after a non-lethal heat shock, indicating Hsp70 has an important role in recovery from stress, even at lower temperatures. Lack of Hsp70 also confers enhanced sensitivity to a temperature-sensitive lethal mutation and to the neurodegenerative effects produced by expression of a human polyglutamine disease protein.

THE heat-shock response, found in all living organisms, provides an effective defense against exposure to adverse environments. The distinctive feature of the heat-shock response is the synthesis of a set of conserved heat-shock proteins (Hsp's; Lindquist 1986; Lindquist and Craig 1988; Parsell and Lindquist 1993; Feder and Hofmann 1999). Hsp's can protect against a number of cellular stresses, including high temperatures, oxidative stress, and a variety of cytotoxins (Craig 1985; Lindquist 1986; Bond and Schlesinger 1987; Pauli *et al.* 1992; De Maio 1995; Morimoto *et al.* 1997; Feder and Hofmann 1999; Jaattela 1999; Kregel 2002). Convincing evidence for the protective function of Hsp's is derived from the induced thermotolerance test. The ability to survive severe heat stress is increased if an organism is first exposed to a mild heat treatment, but not if protein synthesis is blocked (McAlister and Finkelstein 1980; Plesofsky-Vig and Brambl 1985). Such mild heat treatments offer protection against a variety of stresses. The generally accepted interpretation for this phenomenon is that the synthesis of Hsp's that is triggered by the mild heat shock aids subsequent survival under more severe or alternative stresses.

The Hsp's and their constitutively synthesized relatives (termed heat-shock cognates, or Hsc proteins) form a diverse group of protein chaperones that can disaggregate proteins from large aggregates or assemblies, prevent aggregation of denatured proteins, aid the renaturation or folding of proteins to reach their proper conformation, direct proteins to degradative pathways, and bind proteins to restrain their function, making them available for ligand binding or allowing them to translocate across membranes (Glover and Tkach 2001; Houry 2001; Ryan and Pfanner 2001; Walter and Buchner 2002; Craig 2003; Newmyer *et al.* 2003; Pratt and Toft 2003). Although some of the classes of Hsp's clearly have distinct activities, they also exhibit overlapping functions (Sanchez *et al.* 1993), cooperate in their activities (Smith 1993; Ehrnsperger *et al.* 1997; Lee *et al.* 1997; Glover and Lindquist 1998; Veinger *et al.* 1998; Lee and Vierling 2000; Cashikar *et al.* 2005; Haslbeck *et al.* 2005), and may share proteins that act as cofactors, known as cochaperones (S. Chen *et al.* 1996; Glover and Lindquist 1998; Abbas-Terki *et al.* 2001).

Among Hsp's, Hsp70 is ubiquitous, with unusually high conservation in protein sequence and functional features (Boorstein *et al.* 1994). The chaperone functions of the Hsp70 family of proteins are well established (Skowyra *et al.*, 1990; Flynn *et al.*, 1991; Schroder *et al.*, 1993; Hartl, 1996; Hartl and Hayer-Hartl, 2002; Mayer and Bukau 2005). Hsp70 and its relatives have several other roles as well. Although Hsp70 is not itself a protease, it is now known that cochaperones can control its activity to direct substrate proteins either to refold or to be degraded. CHIP (carboxyl-terminus of

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Hsc70 interacting protein) is a ubiquitin ligase that associates with Hsp70 and Hsp90 and their non-stress-induced cognates to direct substrate proteins to the proteasome (Ballinger *et al.* 1999; Connell *et al.* 2001; Höhfeld *et al.* 2001; Meacham *et al.* 2001; Murata *et al.*, 2001; McDonough and Patterson 2003). CHIP is also involved in regulation of the heat-shock response (Dai *et al.* 2003). Other activities of Hsp70 family proteins are the regulation of apoptosis and eliciting innate and adaptive immunity (Gabai *et al.* 1997; Jaattela *et al.* 1998; Beere *et al.* 2000; Wadhwa *et al.* 2002; Wallin *et al.* 2002; Takayama *et al.* 2003; Gullo and Teoh 2004). Interest in the Hsp70 class of chaperones is growing because of the large variety of cellular processes in which they are involved, as well as their possible participation in aging, cancer, and several neurodegenerative genetic disorders.

The heat-shock response was first discovered in Drosophila, as a change in the puffing pattern of salivary gland polytene chromosomes in response to heat (Ritossa 1962). In Drosophila this response is particularly dramatic: following a shift to high temperature nearly all transcription and translation is devoted solely to expression of *Hsp* genes, while other genes are turned off (McKenzie *et al.* 1975; Spradling *et al*, 1975; Storti *et al.* 1980; Scott and Pardue 1981). Hsp70 is the major protein synthesized during this period. Although the synthesis of Hsp70 is nearly undetectable in Drosophila cells at the normal growth temperature of 25°, its expression is rapidly induced at least 1000-fold by raising the temperature to 37° (Velazquez *et al.* 1983). The prominent expression of Hsp70 suggests that it may play a large role in thermotolerance, and experimental evidence confirms this supposition. Solomon *et al.* (1991) found that Drosophila cells with extra copies of *Hsp70* genes had increased survival after heat shock, but that interference with Hsp70 reduced their survival. Additionally, when cells carrying a *metallothionein*-controlled *Hsp70* gene were treated with copper at normal temperature, and then shifted directly to severe temperature, a dramatic increase in survival was observed. Additional transgenic copies of *Hsp70* provide Drosophila with enhanced thermotolerance (Welte *et al.* 1993; Feder *et al.* 1996; Krebs and Feder 1998). However, long-term survival is reduced by extra copies of *Hsp70* (Krebs and Feder 1997, 1998). Thus, the role of *Hsp70* in Drosophila thermotolerance is still not fully understood. As a counterpoint to the Drosophila results implicating Hsp70 in thermotolerance, both *Saccharomyces cerevisiae* and *Escherichia coli* rely mainly on the Hsp100 family to survive severe temperatures (Squires *et al.* 1991; Parsell and Lindquist 1993; Sanchez *et al.* 1993); however, Hsp100 has not been found in animals (Glover and Tkach 2001).

In addition to its role in the Drosophila heat-shock response, Hsp70 and its cognates are clearly involved in non-heat-shock processes. A recent significant discovery is the finding that Hsp70 can modulate the effects of expressing polyglutamine disease genes. Polyglutamine (polyQ) diseases are a group of dominant inherited neurodegenerative disorders of humans, with the disease alleles characterized by expanded segments of CAG repeats encoding polyglutamine (Paulson *et al.* 2000). The pathogenic polyQ proteins are thought to self-associate to form insoluble aggregates inside cells, termed intracellular inclusions. When a segment of the human Machado–Joseph disease gene, which included a segment of CAG repeats, was expressed in the fly eye, developmental defects such as rough eyes and loss of pigment cells and photoreceptor neurons were observed (Warrick *et al.* 1998). It was further shown that Hsp70 was located in the aggregates, that overexpression of human *Hsp70* suppressed the disease, and expression of a dominant negative mutant of a constitutively expressed *Hsp70-cognate* gene (*Hsc4.k71*) enhanced the degeneration (Warrick *et al.* 1999). Drosophila appears to be a very useful model organism to study human polyQ diseases and other human neurodegenerative diseases, and to uncover the role of Hsp70 in those pathologies (Feany 2000; Bonini and Fortini 2003).

In the work that we report here, we made use of Drosophila *Hsp70* deletion mutants to study the role of Hsp70 in thermotolerance and the regulation of the heat-shock response, protein folding, and neurodegeneration. We find that the *Hsp70* mutants affect all these processes.

MATERIALS AND METHODS

Drosophila strains and culture conditions: The names of the *Hsp70* deletion mutants (Gong and Golic 2004) have been altered slightly to conform to FlyBase convention (http://flybase.bio.indiana.edu/). Each homologous recombination event introduced a w^{hs} marker gene. The alleles carrying these w^{hs} markers were used in all experiments, except the polyQ experiments and the heat-shock puff analyses, in which the w^{hs} markers were removed by Cre-mediated recombination prior to the tests (Siegal and Hartl 1996). Fly lines bearing *gmr-GAL4* and *UAS-MJDtr-Q61* were provided by N. M. Bonini (Chan *et al.* 2000). Fly lines carrying *Hsp70* transgenes were provided by M. E. Feder (Welte *et al.* 1993). The shi^{1} flies were obtained from Drosophila stock center (Bloomington, IN). Flies were raised at 25° on standard cornmeal–agar medium and crosses were carried out in standard vials or bottles.

Fertility tests: To test fertility, 10 vials per genotype were started with two females and three males per vial. Flies were transferred to fresh food every day for approximately the first three weeks after the crosses were started, and thereafter every 1–6 days. Progeny eclosing from all vials were scored and summed as a measurement of lifetime fertility. The numbers reported reflect average lifetime fertility of 2×3 matings. The genotypes tested were w^{1118} (12 copy), w^{1118}; *Df(3R)Hsp70A Df(3R)Hsp70Ba*^{304} (6 copy) and w^{1118}; *Df(3R)Hsp70A Df(3R)Hsp70B* (*Hsp70*-null).

Heat-shock protocols: The heat-shock protocols used in these experiments were empirically derived. For the adult heat-shock experiments, 0–1-day-old flies were anesthetized and grouped as 25 adult males or females per vial. On the next day, these 1–2-day-old adult flies were transferred to empty

25 × 75 mm glass vials, given a mild heat shock at 35° for 30 min, and then immediately transferred to 39°. Heat shocks were given by immersing the vials in a circulating water bath as described (Golic and Lindquist 1989). Every 10 min flies were checked under the microscope, then quickly returned to the 39° water bath. If flies did not move any parts of their bodies, even after vials were tapped, they were counted as paralyzed. The results for males and females were very similar, and so no distinction is made in reporting the results.

For the larval thermotolerance experiments, food that contained larvae was immersed in 0.7% NaCl to induce larvae to leave the food. Third instar larvae were collected manually and transferred to a drop of yeast paste on a coverslip, which was then placed into a new vial with fresh food at a concentration of 40 larvae per vial. The vials were heat-shocked at 35° for 30 min, then immediately shifted to 39° for 45 min. The number of eclosing flies was normalized to that without heat-shock treatment. In a second heat-shock protocol third instar larvae were treated at 37° for 1 hr, with or without a 30-min 35° preheat shock.

In the shi^{1} experiments 0–1-day-old adult flies were anesthetized. On the next day these 1–2-day-old adult flies were transferred to empty glass vials, given a pretreatment at 35° for 30 min, and then heat-shocked at 38° for 40 min. At 15-min intervals, for 2 hr after return to room temperature, recovery from paralysis was scored. If a fly could stand after the vial was tapped it was scored as having recovered. Flies were then transferred to the vials with food, and survival was scored the next day.

Cytology: Third instar larvae were heat-shocked at 37° for 25 min. Then at various times after return to room temperature salivary gland polytene chromosomes were prepared as described (Lefevre 1976). For each nucleus, we examined the heat-shock puffs at 63B, 67B, 93D and 95D, representing the major heat-shock-inducible loci: *Hsp83*, the small *Hsp* genes, *Hsrω* and *Hsp68*. When no heat-shock puffing was observed at any of the four loci a nucleus was scored as showing no puffing; if any of the heat-shock puffs were visible it was scored as exhibiting heat-shock puffing.

Statistics: Statistical analyses were performed using Graphpad Prism and Instat software for Macintosh. The lifetime fertility tests were analyzed using the Mann-Whitney test, with the lifetime production from each 2 × 3 mating treated as a single datum, grouped by genotype. The thermotolerance tests of Figure 1A were analyzed by two-factor ANOVA. The results presented in Figures 1B and 2, A–C, were analyzed using the Mann-Whitney test. In the adult tests a pair of vials (consisting of 25 flies of each sex for a total of 50 flies) was treated as a separate datum at each time point. In the larval tests each vial of 40 larvae was treated as a separate datum. Results were grouped by genotype for analysis. The error bars in Figures 1, 2, and 4 represent ±1 SEM. Error bars are not visible for many of the points on the line graphs because they are smaller than the symbols used to represent the data points. The *Hsp* gene repression experiment of Figure 3A was analyzed using a paired *t*-test.

RESULTS

The *Hsp70* deletion genotypes: *Drosophila melanogaster* carry six copies of *Hsp70* per haploid genome, situated at two closely linked loci on chromosome *3*. Deletion mutants were generated by homologous recombination (Gong and Golic 2004). The deletion of the two *Hsp70* genes at the 87A locus is called *Df(3R)Hsp70A*, the single gene *Hsp70Ba* deletion allele is called $Hsp70Ba^{304}$, and the four-gene deletion of all *Hsp70B* genes is called *Df(3R)Hsp70B*. By combining *Df(3R)Hsp70A* with $Hsp70Ba^{304}$ we produced a chromosome lacking three of the six copies normally found on that chromosome; combining *Df(3R)Hsp70A* with *Df(3R)Hsp70B* produced a chromosome completely lacking *Hsp70*. All the mutant combinations were viable and fertile as homozygotes, including the *Hsp70A Hsp70B* recombinant that completely eliminates *Hsp70*, and all had similar developmental times (Gong and Golic 2004; not shown).

In a measurement of lifetime fertility, by crossing *inter se*, we found no significant difference between the 12-copy w^{1118} controls and the 6-copy flies (353 progeny *vs.* 284 progeny, $P = 0.25$). The *Hsp70*-null flies did produce fewer progeny in our tests (105, $P < 0.002$). The reduced fertility of flies lacking *Hsp70* appears to be attributable primarily to a reduction in the progeny produced by females, with male fertility unaffected (not shown).

Reduced thermotolerance of *Hsp70* mutants: We first determined whether *Hsp70* deficiencies had an effect on thermotolerance of adult flies. A standard thermotolerance assay is to first give flies a mild heat shock to allow the synthesis of Hsps, then test the ability of the pretreated flies to tolerate extreme temperatures. Flies were pretreated at 35° for 30 min, then moved to 39° and assayed for their resistance to the paralysis that is a consequence of exposure to high temperatures. We found that a reduction of *Hsp70* copy number makes flies more susceptible to heat paralysis (Figure 1A). Wild-type flies, having 12 copies of *Hsp70*, can withstand 50–60 min at 39° before half of the flies are paralyzed; flies with only eight copies reach this point after ~40 min of exposure ($P < 0.0001$); and flies with six copies require only ~30 min to reach the same level of paralysis ($P < 0.0001$ in comparison with wild-type; $P < 0.0001$ in comparison with eight-copy flies).

Surprisingly, flies that completely lacked *Hsp70* were just as resistant as the flies with six copies of *Hsp70*. However, further examination revealed that having six copies of *Hsp70* clearly provided a survival advantage compared to flies without *Hsp70*. We heat-shocked adult flies at 35° for 30 min, then at 39° for 40 min, and scored their overnight survival after being returned to 25° (Figure 1B). The flies with 12 or six copies of *Hsp70* showed similar rates of survival (81% and 72% respectively, $P = 0.18$), but the flies without *Hsp70* had a greatly reduced survival (32%; $P < 0.0001$). We note that this result also indicates that the majority of six-copy flies that showed paralysis at the 40 min time point in Figure 1A will recover and survive. *Hsp70* appears to have two roles in adult thermotolerance: a high copy number is needed to provide rapid resistance to the effects of severe heat shock, but a lower copy number is sufficient for long-term survival.

To determine whether reduced *Hsp70* copy number also affects larval thermotolerance we measured larva-to-adult survival after applying a 35° 30-min pretreatment followed immediately by a 39° 45-min heat shock to

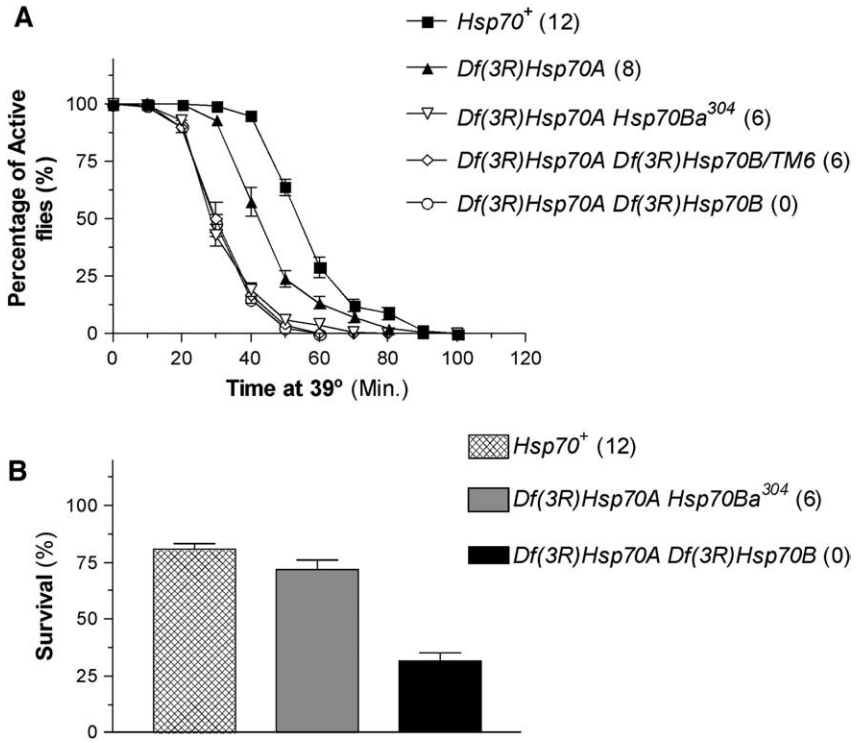

FIGURE 1.—Adult thermotolerance. (A) The rates at which flies of different genotypes succumb to heat shock are shown. The numbers in parentheses indicate the *Hsp70* copy number of each genotype. (B) Overnight survival of flies with different doses of *Hsp70* genes. Heat-shock conditions are discussed in the text. The sample size for each genotype was 300–500 flies. Genotypes are as indicated, except that all flies also carried the w^{1118} mutation on their *X* chromosomes.

third instar larvae. The survival rates were determined by normalizing the frequency of eclosion to that obtained without heat shock. The results were similar to those for adult survival (Figure 2A). A small but significant reduction in viability was observed for flies with only six copies of *Hsp70* ($P < 0.0001$), while *Hsp70*-null flies rarely survived this treatment ($P < 0.0001$). To show that this lack of thermotolerance was specifically attributable to *Hsp70* we added 12 transgenic copies of *Hsp70* (WELTE *et al.* 1993) to the six-copy flies and to the *Hsp70*-null flies. Wild-type or near-wild-type levels of thermotolerance were restored, indicating that the heat-shock lethality results from the *Hsp70* deficiency.

Hsp70 is not absolutely required to survive heat shock. We subjected larvae to a less severe 37° 60-min heat shock, and found very little difference in survival between wild-type and *Hsp70*-null flies (Figure 2B), regardless of whether the larvae were given a 35° pretreatment ($P = 0.05$) or not ($P = 0.05$). However, the 37° heat shock did cause a significant developmental delay (Figure 2C) for both wild-type ($P < 0.0001$) and *Hsp70*-null ($P < 0.0001$) larvae, and this delay was longer for the *Hsp70*-null than for the wild-type larvae ($P = 0.014$). A pretreatment of 35° for 30 min nearly eliminated this delay in wild-type larvae, but had no effect on the delay in the *Hsp70*-null larvae, identifying *Hsp70* as a crucial component in eliminating developmental delay. This result confirms and complements the previous finding of WELTE *et al.* (1993), who showed that extra copies of *Hsp70* could alleviate a heat-shock-induced delay in embryonic development. Although *Hsp70* is not vital at the reduced heat shock used here, it clearly still serves an important function.

The observation that pretreatment had essentially no effect on the survival or developmental delay of *Hsp70*-null larvae heat-shocked at 37° for 60 min led us to ask whether acquired thermotolerance, the increase in stress resistance produced in response to the pretreatment, was entirely dependent on *Hsp70*. To make this determination we gave third instar larvae the same 39° 45-min heat shock as before, but without pretreatment (Figure 2A). Only approximately one-third (34.2%) of the 12-copy larvae survived this heat shock, clearly showing that the pretreatment, with 72.3% survival, provided a large benefit to $Hsp70^+$ larvae. The same heat shock caused almost complete lethality to *Hsp70*-null larvae (0.8% survival). Although the pretreated larvae showed some benefit from pretreatment, their survival was still quite low (7.1%). We conclude that the remaining Hsp's can provide some degree of acquired thermotolerance on their own, but in the absence of Hsp70 their contribution is quite small.

Repression of the heat-shock response is delayed in *Hsp70*-null flies: The developmental delay observed in *Hsp70*-null larvae indicates that Hsp70 aids in the recovery from heat shock to allow more rapid resumption of normal development. It has been previously proposed that Hsp70 is a major regulator of heat-shock response repression during recovery (DIDOMENICO *et al.* 1982a,b; SHI *et al.* 1998; MARCHLER and WU 2001). To test whether the developmental delay observed in *Hsp70*-null larvae is a consequence of an extended heat-shock response we assayed the course of the transcriptional response by scoring the regression of heat-shock puffs, which reflect *Hsp* gene transcription, on salivary gland

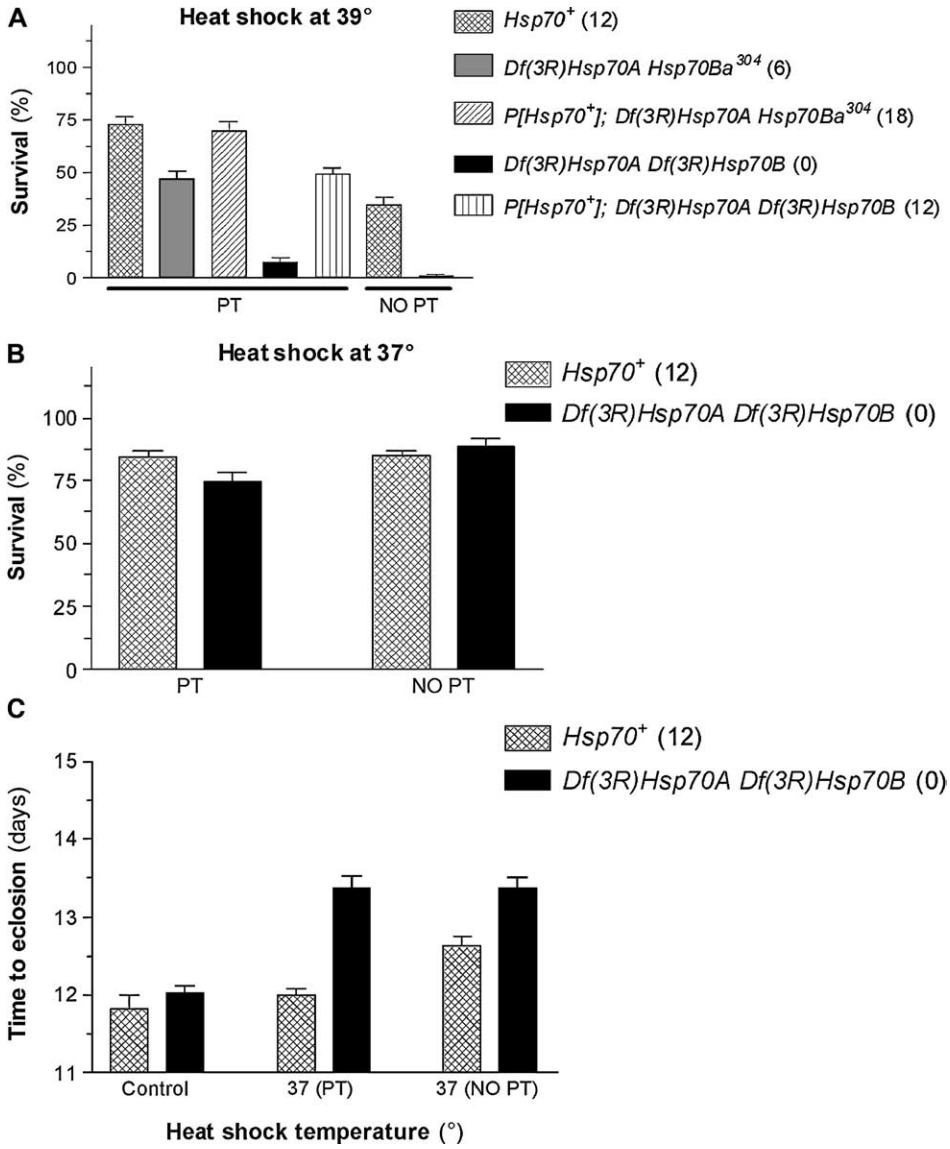

FIGURE 2.—Larval thermotolerance. Third instar larvae of the indicated genotypes were heat-shocked as described in the text, then scored for survival to adulthood (eclosion). The numbers in parentheses represent *Hsp70* copy number. Each bar represents between 360 and 640 larvae. (A) Survival following a 39° heat shock, with (PT) or without (NO PT) pretreatment. *P[Hsp70$^+$]* indicates a *P* element carrying 6 copies of *Hsp70$^+$*, adding 12 copies when homozygous, as is the case here. The NO PT genotypes tested here were *Hsp70$^+$* and *Df(3R)Hsp70A Df(3R)Hsp70B* (the left and right NO PT bars, respectively). (B) Survival following a 37° heat shock. (C) Length of development (egg to adult) in response to a heat shock given to third instar larvae. The control represents developmental time for non-heat-shocked flies. All genotypes are as indicated, except that all flies carried either *w* or *w^{1118}* on their *X* chromosomes.

chromosomes. We heat-shocked third instar larvae at 37° for 25 min, and at various times afterwards we monitored the presence of heat-shock puffs. For each time point and each genotype >50 nuclei were scored. Nuclei were scored as positive if any active heat-shock puffs were observed. We found that the heat-shock puffs persist much longer in the *Hsp70*-null strain, with puffs disappearing in half of the nuclei of wild-type larvae by 40 min, but requiring over 70 min to reach the same point in the *Hsp70*-null larvae ($P < 0.01$, Figure 3).

The extended transcription of heat-shock genes in this experiment is clearly insufficient to account for the 1.5-day delay in development in the previous experiment. Part of the explanation likely lies with the fact that we used a lesser heat shock in this experiment (25 min *vs.* 60 min previously), and the delay in recovery may well be longer with a stronger heat shock. It is also likely that the regression of heat-shock puffs tells only part of the story. The response to heat shock in *Hsp70*-null flies is clearly defective, and this is likely to have consequences for the animal for some time after the transcriptional response has been repressed (see DISCUSSION).

Loss of *Hsp70* enhances a temperature-sensitive mutant phenotype: If the major function of Hsp70 in response to heat shock is to aid the refolding of proteins that have been denatured by exposure to high temperatures, then it is likely that the reduced thermotolerance of *Hsp70*-null flies results from a loss of this ability. To test more directly whether loss of Hsp70 inhibits the recovery of function for proteins affected by heat shock, we examined flies carrying a temperature-sensitive (ts) mutation in *shibire* (*shi*), which encodes dynamin, a protein involved in synaptic vesicle recycling. The *shi^1* allele has a single amino acid substitution in the GTPase domain (VAN DER BLIEK and MEYEROWITZ 1991; DAMKE *et al.* 1995). Exposure to the nonpermissive temperature causes rapid and reversible paralysis (GRIGLIATTI *et al.* 1973; OZAWA and HAGIWARA 1976; SIDDIQI and BENZER

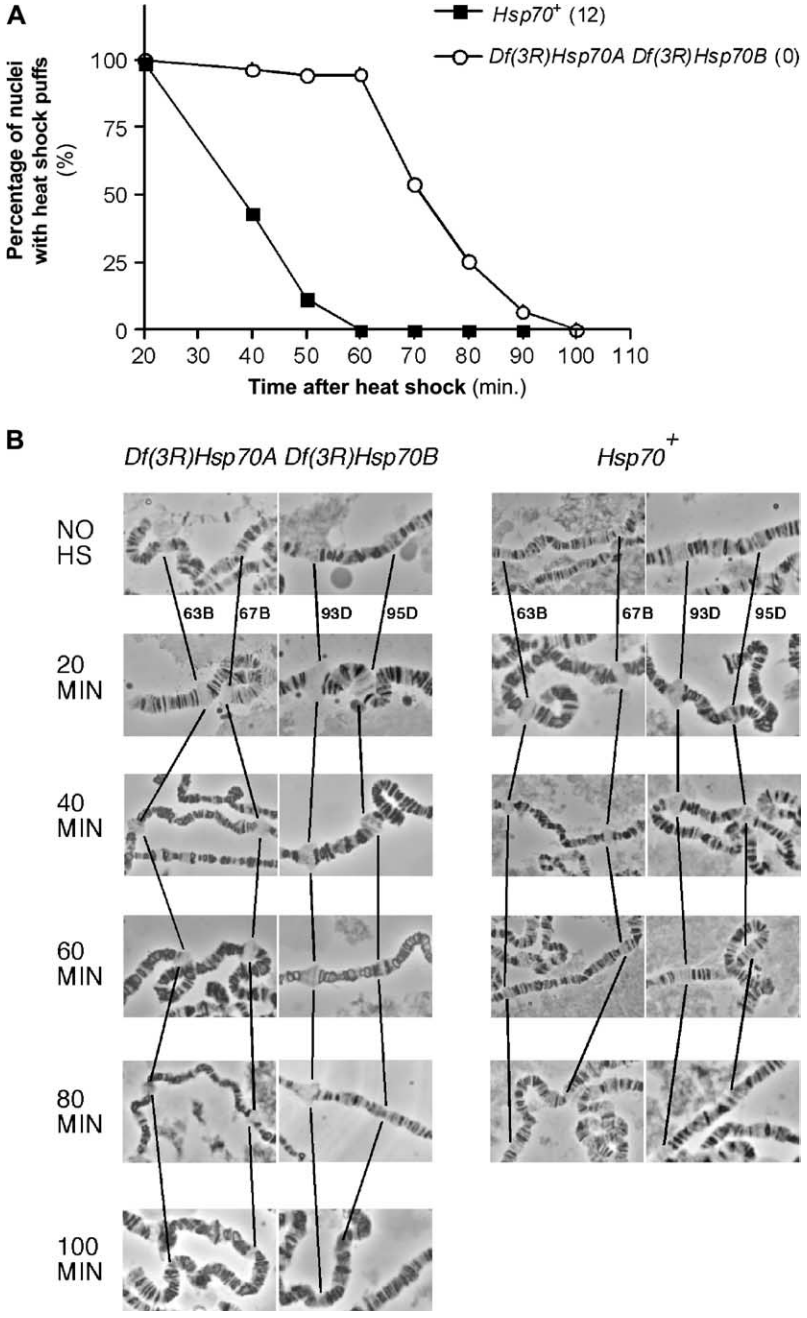

FIGURE 3.—The effect of *Hsp70* on repression of the heat-shock response. The results of assaying heat-shock puff regression in *Hsp70*$^{+}$ and *Hsp70*-null larvae at various times during recovery from heat shock are shown. (A) The percentage of nuclei exhibiting heat-shock puffing. (B) Representative puffing patterns at various times after heat shock. The cytological locations of other heat-shock puffs are indicated. Experimental details are given in the text. Genotypes are as indicated, except that all flies carried w^{1118} or *w* on their *X* chromosomes.

1976; KOSAKA and IKEDA 1983; KAWASAKI *et al.* 2000). Because *shi*^{1} flies have an easily scored temperature-sensitive phenotype, and because this phenotype is reversible upon return to normal temperature, we thought this would be a good test of the participation of Hsp70 in facilitating the recovery of protein function after heat shock.

Flies were pretreated at 35° for 30 min and then given a 40-min heat shock at 38°. We scored recovery from paralysis at several time points during a 2-hr recovery at room temperature (Figure 4A). The *Hsp70*-null flies were unaffected by this heat treatment. The *shi*^{1} flies were initially paralyzed, but showed nearly complete recovery after ~30 min at room temperature. The *shi*^{1} *Hsp70*-null double mutants were extremely sensitive, with the majority failing to recover within the 2-hr period. No further recovery was observed when these flies were examined again the next day (Figure 4B). Thus, without Hsp70, the effect of heat on *shi*^{1} is effectively irreversible. Because *shi*^{1} flies that do have *Hsp70* recover rapidly after heat shock, it is unlikely that recovery of *shi*^{1} flies from heat shock requires new synthesis of shibire/dynamin. Instead, we consider it most likely that the preexisting dynamin reacquires a functional conformation, and that this process is strongly dependent on direct interaction with Hsp70 that facilitates refolding.

***Hsp70* deficiencies enhance polyQ cellular degeneration:** Another experimental paradigm with strong

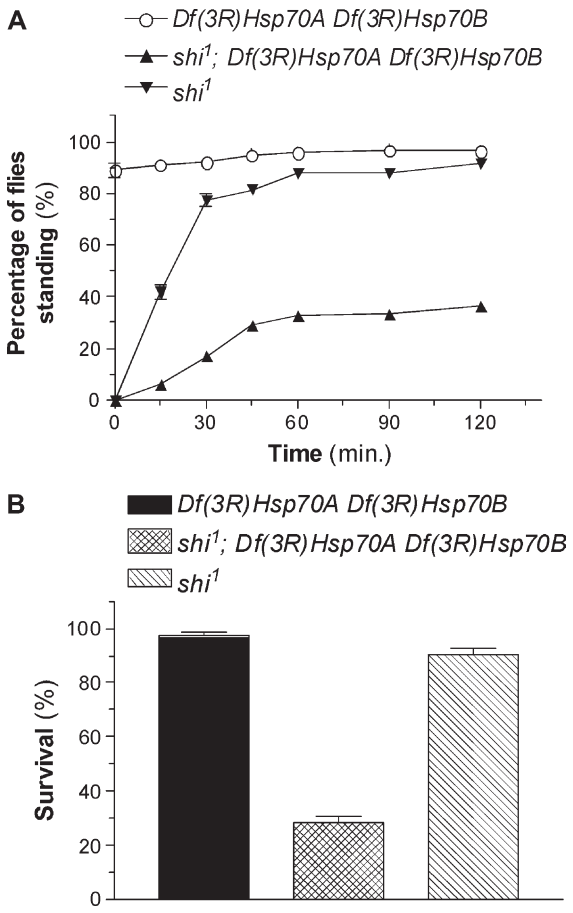

FIGURE 4.—The effect of *Hsp70* on the temperature-sensitive paralytic mutations, *shi*^{1}. (A) Recovery from heat-shock-induced paralysis, scored at intervals following return to room temperature. (B) Survival after overnight recovery at room temperature. Experimental details are given in the text. Approximately 350 flies were scored for each genotype, with 23–29 of each sex in each vial. Genotypes are as indicated, except that *shi*$^{+}$ flies carried *w*^{1118} on their *X* chromosomes and the *shi*^{1} flies carried *w*$^{+}$.

involvement of Hsp70 chaperone function is the cell death brought about by expression of a human neurodegenerative disease protein (BONINI 2002). Defects in the human *MJD1* gene are responsible for Machado–Joseph disease (MJD), or Spinocerebellar Ataxia type 3 (KAWAGUCHI *et al.* 1994). This gene has a polyQ tract, and alleles with polyQ expansions act dominantly to produce neurodegenerative phenotypes. WARRICK *et al.* (1998) constructed transgenic flies for eye-specific expression of a truncated form of MJD1 (*MJDtr*) with an expanded polyQ repeat. Degenerative phenotypes such as rough eyes and loss of pigment cells were observed. Several studies have implicated chaperone function in such neurodegenerative diseases (BONINI and FORTINI 2003). For instance, overexpression of *Hsp70* in the Drosophila eye alleviates the degenerative phenotypes produced by *MJDtr* expression, and dominant negative forms of Hsc70, or of Hsp70 cochaperones, enhance the degenerative phenotypes.

To test whether reduction in *Hsp70* dosage would affect the *MJDtr* phenotypes we combined the *Hsp70* deletions with transgenes that expressed *MJDtr*. We chose to test a combination that produced a relatively mild neurodegenerative phenotype by itself because we expected that loss of *Hsp70* would enhance the phenotype. We found that this was so, and the observed enhancement was roughly proportional to the number of copies that were deleted (Figure 5). In the combinations having the fewest copies of *Hsp70* the phenotype was notably less severe in the anterior of the eye, likely reflecting the fact that cells differentiate later in the anterior than in the posterior and have expressed *MJDtr* for a shorter time than cells in the posterior. Our results confirm that the normal complement of wild-type *Hsp70* genes plays a role in mitigating the damage that results from expression of an expanded polyQ disease protein.

DISCUSSION

Flies with a reduction in *Hsp70* copy number are viable, including flies with no copies of *Hsp70*. Similarly, mice that lack all *Hsp70* genes are also viable (HUNT *et al.* 2004). However, mutations in several of the constitutively expressed homologues of *Hsp70* in Drosophila do cause lethality (ELEFANT and PALTER 1999; BURMESTER *et al.* 2000; http://flybase.bio.indiana.edu), indicating that the Hsc70 family of proteins carries out critical functions at normal temperatures. Furthermore, we found that *Hsp70*-null females, though fertile, have a significant reduction in fertility, indicating that the heat-inducible genes also have some role at normal temperature. Nonetheless, the fact that the Drosophila *Hsp70* deletion strains are viable and fertile as homozygotes provided substantial versatility in characterizing the effects of *Hsp70* dosage, and also allowed us to examine the phenotypes of *Hsp70*-null flies.

When multiple copies of a gene are present, the possibility that those genes have divergent functions must be considered. For instance, in yeast, the *SSA1* and *SSA2* genes, though encoding nearly identical Hsp70 proteins, differ in their interaction with the [URE3] prion (SCHWIMMER and MASISON 2002). LAKHOTIA and PRASANTH (2002) presented evidence that the two *Hsp70* clusters of *D. melanogaster* are differentially regulated. However, BETTENCOURT and FEDER (2002) concluded that the *Hsp70* gene copies have not diverged, and likely all provide the same function. The proteins encoded by these six genes have between 98.6% and 100% identity, and 99.5% or greater similarity. In the work we present here, we have assumed that the deletions we produced are functionally distinguished only by copy number. Our experiments on thermotolerance and the effect of polyQ protein expression are easily interpreted strictly in terms of *Hsp70* copy number. For instance, in the adult thermotolerance experiment, the two different six-copy genotypes were indistinguishable. Furthermore,

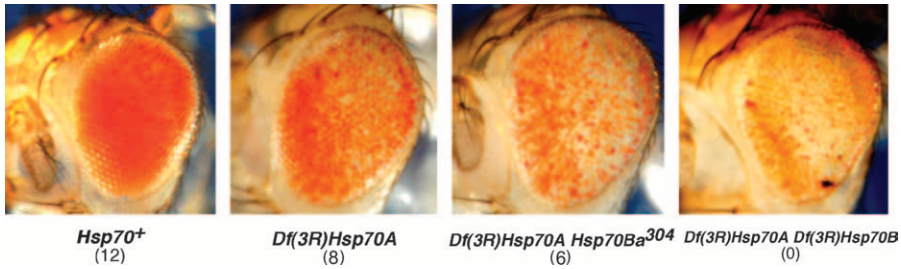

FIGURE 5.—The effect of *Hsp70* on neurodegeneration produced by expression of a human disease gene. As shown here, a reduction in *Hsp70* copy number enhances the cell death and pigment loss that is produced by expression of *MJDtr* in the eye. Representative examples of the phenotypes are shown. With the wild-type *Hsp70* copy number, only slight pigment loss is observed (left), while a complete lack of *Hsp70* strongly enhances the phenotype (right). In addition to the *Hsp70* genotypes indicated, with the *Hsp70* copy number indicated in parentheses, all flies were *w; gmr-GAL4 UAS-MJDtr-Q61/SM1*.

when transgenic copies of *Hsp70* were added back to the *Df(3R)Hsp70A Hsp70Ba*^{304} genotype, they complemented the larval thermotolerance defect, even though the *Hsp70* transgenes represent genes that were not deleted in that genotype (CRAIG *et al.* 1979; WELTE *et al.* 1993).

The two *Hsp70* clusters differ by intergenic segments. Portions of the *S-element* transposon are found at both 87A and 87C, and the examination of sequence diversity suggests that these elements are maintained by selection (MASIDE *et al.* 2002). However, there are several other *S-elements*, including many complete elements, found throughout the genome, so it seems unlikely that these particular copies provide any function that is not also encoded elsewhere (KAMINKER *et al.* 2002). The large intergenic region at 87C hosts the largest euchromatic cluster of transposons found in the genome (KAMINKER *et al.* 2002), and includes heat-shock-transcribed repetitive DNA, the αβ and αγ repeats (LIS *et al.* 1978). It is not known whether these transcripts serve any function. Their heat-induced transcription at this location may simply be an accident of proximity to the massively induced *Hsp70* genes. Copies of the same sequences found in centric heterochromatin are not induced by heat shock (LIS *et al.* 1981). Although we think it unlikely, we cannot rule out the possibility that deletion of these repetitive sequences plays a role in some of the phenotypes we described. To fully address such questions it may be necessary to introduce point mutations into individual genes, or to delete specific repetitive elements (RONG *et al.* 2002; GONG and GOLIC 2003).

Not surprisingly, we found that Hsp70 makes an important contribution to thermotolerance in Drosophila subjected to a severe 39° heat shock. Adult flies with reduced *Hsp70* copy number succumb more quickly to lethal high temperatures than do flies with their full complement of *Hsp70* genes, larvae are killed at a higher rate, and larvae lag in development in response to nonlethal heat shocks. Many previous studies that examined the effects of *Hsp70* overexpression in cell lines or in whole animals, or underexpression in cell lines, also led to the conclusion that Hsp70 is an important component of thermotolerance (SOLOMON *et al.* 1991; FEDER *et al.* 1996; FEDER and KREBS 1997; KREBS and FEDER 1998; ROBERTS *et al.* 2003). However, none of these prior studies were able to examine the effects of heat on flies that completely lacked Hsp70. Furthermore, some studies show that overexpression of *Hsp70* is not always beneficial. Larvae carrying extra transgenic copies of *Hsp70* have reduced survival following some heat-shock regimens (KREBS and FEDER 1997, 1998). Females with extra copies of *Hsp70* also show a reduction in fertility following heat shock (SILBERMANN and TATAR 2000). Such studies have led to the idea that the existing copy number of *Hsp70* in *D. melanogaster* was produced by a balance between selection for its chaperone function under stress conditions and against its deleterious effects on growth, viability, and fecundity (FEDER and HOFMANN 1999). Thus, it was important to examine the phenotypes of *Hsp70*-null flies to test whether copy number reduction would also have a deleterious effect on thermotolerance. Our results with *Hsp70* mutants confirm that Hsp70 plays a major role in thermotolerance in Drosophila and supports the hypothesis that *Hsp70* copy number represents a balance arrived at by competition between positive and negative selection.

We were surprised to find that Hsp70 is not required to survive a slightly milder 37° 60-min heat shock, even though the temperature was only 2° less than a lethal heat shock. This phenotype of *Hsp70* mutants in Drosophila is reminiscent of the phenotype of *hsp104* mutants in *S. cerevisiae*. Hsp104 is required to tolerate extreme heat shocks, but a small degree of thermotolerance remains in *hsp104* mutants, and at 37°, a temperature that induces the heat-shock response, the mutants grow as well as cells with a functional *HSP104* gene (SANCHEZ and LINDQUIST 1990). The thermotolerance that remains in *hsp104* mutants is attributable to Hsp70 (SANCHEZ *et al.* 1993). The Drosophila strains we characterized in this study still carry the closely related *Hsp68* gene. It is quite possible that the function of Hsp70 is partly provided by Hsp68, much as overexpression of the Hsp70-encoding *SSA1* gene can partially compensate for loss of Hsp104 in yeast (SANCHEZ *et al.* 1993). Indeed, by examining protein

synthesis in embryos homozygous for large deficiencies that removed the *Hsp70* genes (and many other genes as well), Ish-Horowicz *et al.* (1979) found that *Hsp68* expression increased in the absence of *Hsp70*. When *Hsp68* mutants become available it will be informative to combine them with the *Hsp70* mutants to assess phenotypes in the complete absence of this class of heat-induced chaperones. The constitutively expressed forms of Hsp70 may also function at high temperatures to provide a substantial baseline level of thermotolerance. The amount of Hsp70 produced after heat shock is always less than the constitutively synthesized level of Hsc70 proteins (Palter *et al.* 1986), allowing for the possibility that the Hsc70 proteins contribute to thermotolerance, though their expression is not induced by heat shock. A role for *Hsc70* genes in thermotolerance is suggested by results in several organisms (Ulmasov *et al.* 1992; Kampinga 1993; M. S. Chen *et al.* 1996; DiIorio *et al.* 1996).

The heat sensitivity of *Hsp70* mutant flies likely results from the loss of Hsp70 chaperone function and the consequent reduction in the capacity of cells to refold proteins that were denatured by heat shock (Pelham 1986). Evidence for this mechanism is provided by our finding that *shi*^{1} temperature-sensitive paralytic flies, which normally recover rapidly upon return to normal temperature, recover quite poorly if they lack *Hsp70*. Feder and Krebs (1997) showed that *Hsp70* overexpression helped to restore alcohol dehydrogenase activity to *D. melanogaster* larvae after heat shock. Using a luciferase reactivation assay, it has been shown that the Hsp70 homologues from other organisms are also involved in rescuing proteins after thermal denaturation (Schroder *et al.* 1993; Levy *et al.* 1995; Turman and Rosenfeld 1999; Lee and Vierling 2000). Hsp104 performs a similar function in yeast in cooperation with Hsp70 (Glover and Lindquist 1998), and the participation of the Hsp104 homolog in refolding denatured proteins is vital to produce thermotolerance in *E. coli* (Weibezahn *et al.* 2004).

In the absence of Hsp70, proteins that have been unfolded by heat must be refolded by alternate pathways, either spontaneously or with the involvement of other chaperones. The loss of the Hsp70, the most highly expressed Hsp in Drosophila, may overload the remaining chaperone systems and delay recovery. Following a severe heat shock the remaining Hsp's are relatively ineffective, as shown by the only minimal increase in survival that is produced by a low-temperature pretreatment in *Hsp70*-null larvae. The *shi*^{1} experiment shows that the function of some proteins cannot be fully restored without Hsp70. It is likely that they must be synthesized anew to restore function, resulting in developmental delays or lethality. In some cases the sensitivity of genetic screens for temperature-sensitive mutants might be greatly improved by incorporating *Hsp70* deficiencies.

It is conceivable that the temperature-sensitive dynamin encoded by *shi*^{1} may be exceptionally responsive to Hsp70 chaperone activity. The Hsp70 cognate, Hsc70-4, binds to clathrin and dynamin and participates in the assembly and disassembly of clathrin cages, with mutants showing defects in endocytosis and exocytosis (Schlossman *et al.* 1984; Bronk *et al.* 2001; Newmyer and Schmid 2001; Chang *et al.* 2002; Newmyer *et al.* 2003). In *Hsp70*$^{+}$ flies, perhaps Hsp70 takes the part of Hsc70-4 and associates with clathrin and/or dynamin at high temperature. In *shi*^{1} animals these associations could maintain ts-dynamin in a configuration that allows it to resume its function when the temperature is lowered. In the absence of Hsp70, this association does not occur and a change in the conformation of the temperature-sensitive shi^{1} protein may be irreversible. In support of the idea that Hsp70 may substitute for Hsc70-4, *Hsp70* is abnormally induced in *Hsc70-4* mutant flies when it would otherwise be silent (Elefant and Palter 1999; Bronk *et al.*, 2001). However, if this hypothesis were true it seems that *Hsp70*-null flies should exhibit paralysis under the same conditions that inactivate shi^{1}, and they do not.

Hsp70 has been implicated as having a critical role in regulation of the heat-shock response in prokaryotes (Tilly *et al.* 1983) and in eukaryotes. Originally, a tight correlation between repression of *Hsp70* mRNA translation and resumption of non-*Hsp* mRNA translation was observed (DiDomenico *et al.* 1982a,b). Subsequently, interference with *Hsp70* expression was seen to cause a delay in repression of *Hsp* mRNA translation and resumption of normal translation (Solomon *et al.* 1991). In *S. cerevisiae*, the Hsp70-encoding *SSA1* gene similarly carries out self-regulation (Stone and Craig 1990). Under nonstress conditions, Hsp70 participates in repressing the activity of HSF, the positive transcription factor for *Hsp* genes (Halladay and Craig 1995; Shi *et al.* 1998; Marchler and Wu 2001). It is believed that Hsp70, in cooperation with other Hsp's, sequesters HSF and restrains its activity. Under stress conditions the Hsp's are diverted to chaperoning other denatured proteins, freeing HSF to activate transcription of the *Hsp* genes (Morimoto 1998; Voellmy 2004). Our examination of the duration of heat-shock puffing in *Hsp70* wild type and mutant flies confirms that *Hsp70* is needed for normal regulation of heat-shock transcription because *Hsp* repression is delayed in *Hsp70*-null flies.

The functional analysis of Hsp's is certain to be facilitated by the availability of *Hsp70* deficiencies. The absence of Hsp70 is likely to help reveal roles of the remaining Hsp's that were obscured in the presence of Hsp70. Phenotypic assessment of mutant combinations has been quite useful for revealing the roles of specific Hsp-encoding genes in yeast (see, for instance, Craig and Jacobsen 1984; Werner-Washburne *et al.* 1987; Sanchez *et al.* 1993; Parsell *et al.* 1994; Glover and

Lindquist 1998; Cashikar *et al.* 2005; Haslbeck *et al.* 2005). Combining *Hsp70* mutations with mutations in other *Hsp* genes is likely to be equally informative in Drosophila. As we showed, *Hsp70*-deficiencies also sensitize flies to at least one type of neurodegeneration, that resulting from the expression of a human polyQ disease gene. The use of *Hsp70* mutants may facilitate the identification and analysis of other components that either prevent or contribute to such degeneration.

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